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Immunohistochemical Distribution of Type IV Collagenase in Normal, Benign, and Malignant Breast Tissue

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Production of type IV collagenase by tumor cells has been linked to their metastatic potential in several experimental models. A possible role for this enzyme in basement membrane type IV collagen turnover has also been suggested. Two recently developed affinity-purified, monospecific antibodies directed against the amino terminus (H_1), or an internal active site domain (metal binding region [MBR]) of human type IV collagenase, were employed in the avidin-biotin-immunoperoxidase technique in formalin-fixed, paraffin-embedded breast tissue samples from 55 patients. Intense cytoplasmic immunostaining of myoepithelial cells was found in normal and hyperplastic tissue, and discontinuous staining was noted in intraductal carcinomas. Luminal epithelial cells were negative or weakly positive in large- or medium-sized ducts but reacted frequently in normal terminal ducts and hyperplastic lesions. Epithelial cells in intraductal carcinomas exhibited immunoreactivity in 20 of 23 cases. Invasive carcinomas were positive in 36 of 40 cases, and metastatic cells in lymph nodes stained in 10 of 12 cases. These results support a role for type IV collagenase in the basement membrane remodeling of normal breast. Our findings suggest that myoepithelial cells play a pivotal role in this enzymatic activity. The high percentage of positive cells in invasive carcinomas and the strong immunoreactivity of lymph node metastases support the role of the enzyme in tumor invasion and metastasis and suggest that tumor cells are the essential source of the enzyme in these processes. (Am J Pathol 1990, 136:585–592)

During the last decade, increasing attention has been focused on the role of extracellular matrix, especially basement membrane (BM), in the process of tumor invasion and metastasis.[1–7] Although the biochemical mechanisms involved in BM turnover under physiologic conditions remain essentially unknown,[8] it has been established that the BM-degrading properties of tumor cells correlate with their metastatic potential.[9]

Type IV collagen is one of the major components of BM, and it represents the structural scaffolding of these specialized sheets of extracellular matrix. The enzymatic degradation of type IV collagen is specifically initiated by a neutral metalloproteinase, type IV collagenase.[10,11] This enzyme has been found in human tumor cells[12,13] as well as in other normal cell types, such as endothelium,[14] fibroblasts,[13] macrophages,[15] polymorphonuclear leukocytes,[16] and keratinocytes.[17] It is secreted in a latent form that can be activated, at least *in vitro*, by trypsin and organomercurial compounds. This activation is associated with the loss of 80 amino acid residues from the amino terminus.[18]

The structure and changes of BM in breast lesions have been extensively studied,[19–24] and a variable loss of BM is a constant feature in invasive neoplasms. Previous investigators, employing polyclonal antibodies against murine type IV collagenase, have found positive immunoreactivity in invading breast cancer cells but not in normal breast, benign lesions, or *in situ* carcinomas.[25]

We have used two recently developed affinity-purified rabbit polyclonal antibodies directed against synthetic peptides corresponding to different domains of the human type IV collagenase in an attempt to study the immunohistochemical distribution of this enzyme in normal and hyperplastic breast tissue as well as in primary malignant lesions and metastases.

Materials and Methods

Tissues

Breast tissue samples from 55 patients were obtained from the files of the Laboratory of Pathology, National

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Cancer Institute. The tissues were routinely fixed in 10% neutral buffered formalin and embedded in paraffin. Nontumorous tissue included 14 cases of normal breast, six cases of fibrocystic changes, six fibroadenomas, 11 breast lesions showing epithelial hyperplasia (four mild, three moderate, and four florid), and one case of sclerosing adenosis. Forty carcinomas were evaluated: 37 ductal type (34 not otherwise specified, [NOS], two tubular, and one with squamous differentiation) and three lobular. Four cases were classified as pure intraductal carcinoma, but *in situ* tumors were found as a component of 19 invasive cancers. Lobular carcinoma *in situ* was present in all three cases of infiltrating lobular carcinoma. Twelve axillary lymph node metastases from five patients were included in the study.

Frozen tissue samples were also evaluated to compare the reliability of the immunohistochemical results with that of formalin-fixed material.

Antibody Characterization

Anti-type IV collagenase antibodies were prepared with the use of synthetic peptides coupled to bovine serum albumin. Peptide H_1 with the sequence APSPIIKFPGDVAPKTD, corresponding to the 17 amino-terminal amino acid residues of type IV precollagenase, and peptide MBR with the sequence VAAHEFGHAMGLEHSQ, corresponding to the putative metal ion-binding domain of type IV collagenase, were synthesized on a Biosearch 9600 peptide synthesizer (Biosearch, Novato, CA) with tBOC amino acid methodology. The peptides were coupled to bovine serum albumin with glutaraldehyde (0.14%). For the initial immunizations, 1 ml of bovine serum albumin–peptide conjugate was mixed with 1 ml of complete Freund's adjuvant and emulsified prior to subcutaneous injection. For the remaining biweekly immunization, 0.5 ml of bovine serum albumin–peptide conjugate was emulsified with 0.5 ml of incomplete Freund's adjuvant before injection.

Peptide affinity resins were prepared for antibody purification for both antibodies with Affi-Gel 10 (BioRad, Richmond, CA), following the manufacturer's directions and using 2 mg of each peptide. These resins were used to affinity purify the antibodies from rabbit serum, following heat inactivation of the serum at 56 C for 30 minutes. Following absorption of the antibodies onto the resin overnight at 4 C, the columns were washed with 20 column volumes of cold phosphate-buffered saline prior to elution with two bed volumes of 1 mol acetic acid. This eluate was immediately neutralized by the addition of 1 mol NaOH before buffer exchange to phosphate-buffered saline using a YM 10 membrane (Amicon, Danvers, MA).

Purified antibodies H_1 and MBR were stored at 4 C and characterized with Western blot analysis or enzyme-linked immunosorbent assay (ELISA) before use in the immunohistochemical studies. The results of Western blot analyses showed that both antibody preparations were monospecific and reacted with a single species in human melanoma cell (A2058) conditioned media that corresponds to purified type IV collagenase (Figure 1).

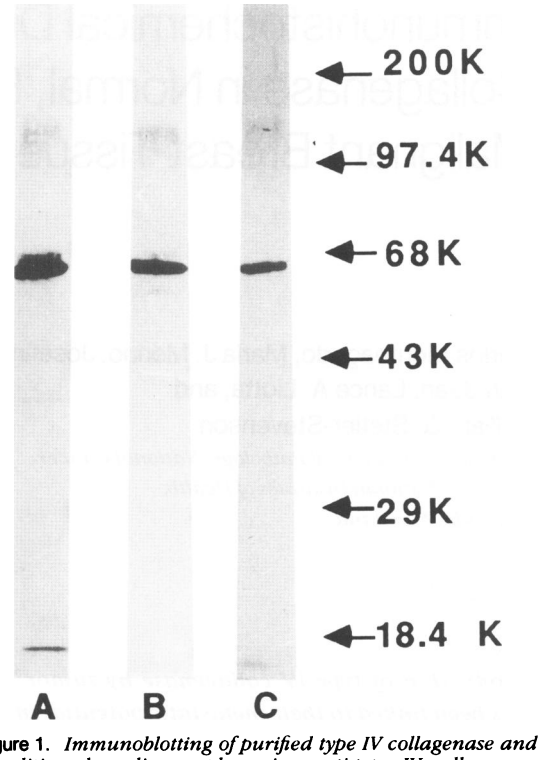


Figure 1. *Immunoblotting of purified type IV collagenase and conditioned media samples using anti-type IV collagenase synthetic peptide antibodies. All samples were run on a 10% polyacrylamide gel using a Laemmli buffer system and electroblotted to Immobilon membranes using a Tris-glycine/methanol transfer buffer system.* **A:** *2 μg of gelatin-affinity purified type IV collagenase. Primary antibody H_1.* **B:** *10 μl of human melanoma cell conditioned media containing approximately 80 ng of type IV collagenase. Primary antibody H_1. The single band indicates that other neutral metalloproteinases present in this sample do not cross react with the primary antibody.* **C:** *10 μl of human melanoma cell conditioned media containing approximately 80 ng of type IV collagenase. Primary antibody MBR. The single band indicates that other neutral metalloproteinases present in this sample do not cross react with the primary antibody.*

Immunohistochemical Procedure

The avidin-biotin-immunoperoxidase technique was used as previously described by Hsu et al.[26] After the sections were dewaxed and rehydrated, endogenous peroxidase activity was blocked by incubating the slides in 0.3% H_2O_2 in absolute methanol for 30 minutes. The tissue samples were then exposed for 20 minutes to 2% normal goat serum. Without washing, first antibodies (H_1 or MBR) were incubated for 45 minutes in a humidity chamber at room temperature. Dilutions were 1 to 2 μg/ml for H_1 and

Table 1. *Distribution of Type IV Collagenase Immunoreactivity in Normal Breast and Benign Breast Lesions*

Tissue*	Myoepithelium†	Luminal/ ductal epithelium†
Normal breast (14)	+++	−/+‡
Benign lesions		
Fibrocystic condition (6)	++	−/+
Epithelial hyperplasia (11)	++	+/++§
Sclerosing adenosis (1)	++	−/+
Fibroadenoma (6)	variable	variable

* Number of cases in parentheses.
† −, no staining; −/+, occasional weak staining; +, weak staining; ++, moderate staining; +++, intense staining.
‡ Mainly in terminal ducts.
§ ++ in florid hyperplasia.

15 μg/ml for MBR. Second biotinylated goat anti-rabbit IgG (dilution 1/200) was incubated for 30 minutes. The slides were then incubated in avidin-biotin-peroxidase (ABP) complex (Vectastain kit, Vector Laboratories, Burlingame, CA) for 45 minutes, and the reaction was revealed by 0.5 mg/ml 3-3'diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) and 3 μl/ml H_2O_2 in 50 mmol TRIS, pH 7.6. All antibodies were diluted in 50 mmol TRIS-buffered saline, pH 7.5. Between steps, the slides were washed three times in TRIS-buffered saline. Control slides were performed by replacing first antibody with the IgG fraction of normal rabbit serum. The sections were considered as positive or negative according to the presence or absence of specific staining and were evaluated on an Olympus microscope (Optical Elements Corp., Washington, DC). The terms "terminal duct" and "ductule" are used here as described by Wellings et al.[27]

Results

The pattern of staining was similar with both antibodies; therefore, we will consider them together except for those cases in which marked differences were noted (Tables 1 and 2). Immunoreactivity appeared as a cytoplasmic, granular, or diffuse staining in all cases. Formalin-fixed as well as frozen tissue showed areas without immunostaining.

Normal Breast

Strong immunostaining of myoepithelial cells (Figure 2A) was found in normal ducts and ductules. This reactivity occurred with both antibodies, but it was more intense with H_1. In areas with involuting changes, such as myoid atrophy, myoepithelium was also strongly stained. Luminal epithelial cells were negative or weakly positive in large- or medium-sized ducts, but in terminal ducts they occasionally showed an enhanced immunoreactivity. In one case of lactating breast associated with an invasive carcinoma, myoepithelial cells were strongly positive, while secretory epithelium was completely negative (Figure 3).

Benign Lesions

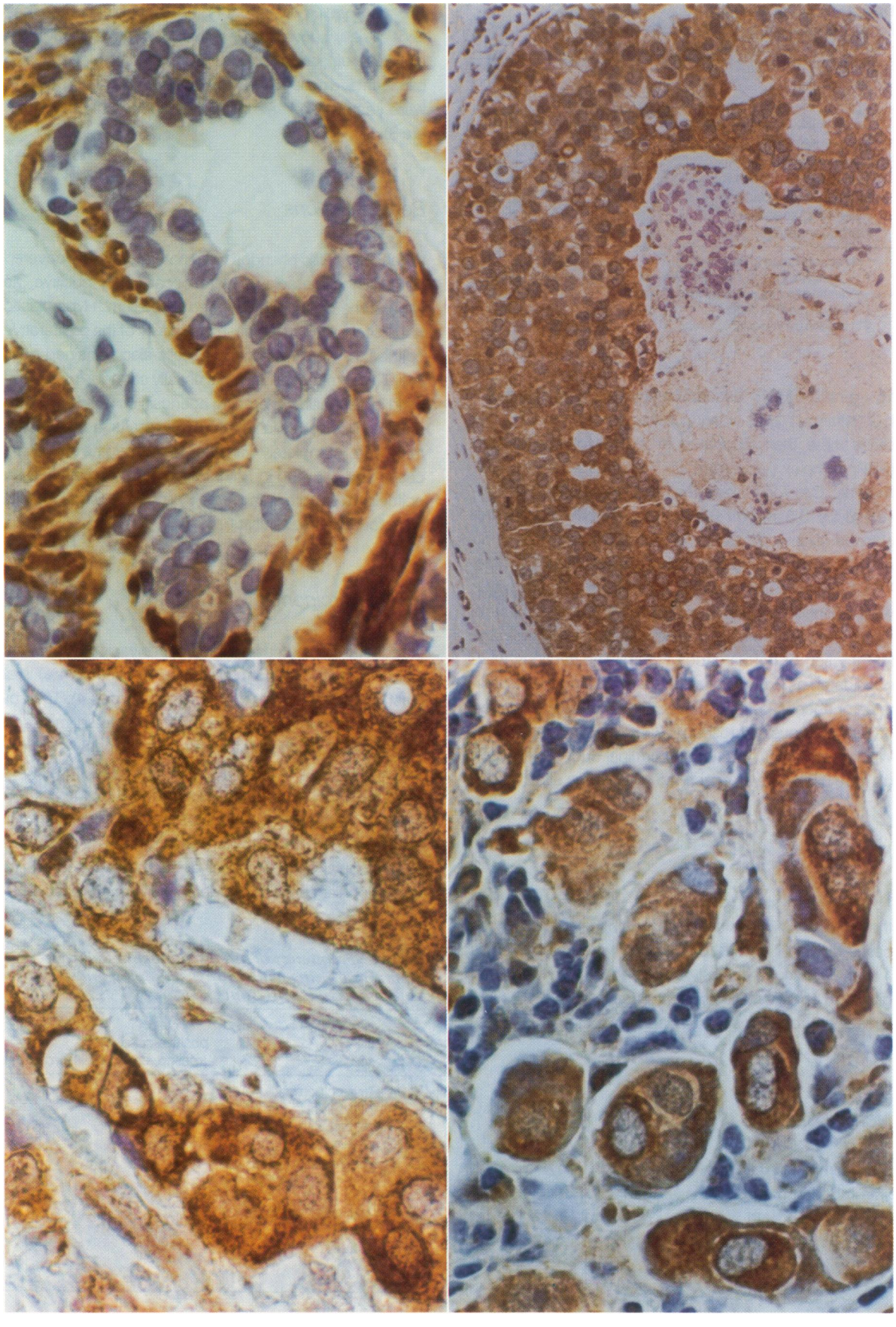
When fibrocystic changes were noted, the cysts covered by apocrine metaplasia were occasionally positive, as were some cysts with flattened epithelium. Hyperplastic lesions revealed an increased reactivity of the proliferative epithelium, while myoepithelial cells also remained positive. The immunostaining was usually stronger in cases of florid hyperplasia. Sclerosing adenosis showed a positive reaction in most cells with H_1. The results in fibroadenomas were completely variable, being negative in some cases and showing indistinct staining of myoepithelium, epithelium, or both in the remaining cases. Similar results were found in frozen tissue.

In Situ *Carcinomas*

Intraductal carcinomas with adjacent invasive component showed positive immunostaining in 16 cases (84%) in at least 10% of the tumor cells (Figures 2B and 4). The epithelial cells in intraductal carcinomas reacted markedly stronger than did those of hyperplastic lesions. A positive, discontinuous, myoepithelial cell layer was present in most cases. Four of four cases of ductal cancer without invasion were positive, and there was no correlation with histologic type (comedo *vs.* noncomedo). Three of three *in situ* lobular carcinomas, all of them associated with invasive lobular carcinomas, were also immunoreactive.

Table 2. *Frequency of Intense Epithelial Cell Immunostaining with Anti-type IV Collagenase Antibodies in Breast Cancer Tissues*

Diagnosis	No. of positive cases/ total no. cases	Percentage
I. *In situ* carcinomas		
Intraductal		
with invasion	16/20	84
without invasion	4/4	100
Lobular, associated with invasion	3/3	100
II. Invasive carcinomas		
Ductal	33/37	89
Lobular	3/3	100
III. Metastases	10/12	83



Invasive Carcinomas

Infiltrating ductal carcinomas were positive in 33 cases (89%) in approximately 80% of the tumor cells (Figure 2C). Invasive lobular carcinomas were also positive in the three cases studied.

Metastases

Metastatic tumor cells in lymph nodes showed immunoreactivity in 10 cases (83%) (Figure 2D). Lymphoid cells were negative, but histiocytes showed occasional positivity of the cytoplasm.

In all samples, smooth muscle cells were immunoreactive. Endothelial cells rarely exhibited a positive staining, mainly in areas of vascular neogenesis. Fibroblasts were mostly negative, although a few immunoreactive ones with myofibroblastlike appearance were found. The frozen tissues stained in a pattern similar to that of the formalin-fixed tissues.

Discussion

The cellular source of extracellular matrix-degrading enzymes has important implications in our understanding of tumor biology and tissue remodeling.[7] During invasion and the process of metastases, tumor cells must traverse epithelial and endothelial BM, where type IV collagen represents a barrier as a major structural component.[9]

Host–tumor cell interactions are known to play a role in the degradation of interstitial (I, II, and III) collagens.[4,28] Pepsinized type IV collagen is not degraded by classical interstitial collagenases,[29] although it is sensitive to some extent to other nonspecific proteases such as elastase, cathepsin G, and plasmin.[3] The isolation and characterization of type IV collagenase[10,11] introduced a new approach in BM physiology and pathology. The role of this enzyme in tumor invasion has been discussed previously.[1,2,6,30] Experimental evidence of a link between the increased production of type IV collagenase by tumor cells and metastatic behavior was described in murine models,[9] and recently it has been extended to the *ras*-induced metastatic phenotype.[31]

Our findings of a marked increase in type IV collagenase immunoreactivity in invasive breast carcinomas are in accordance with previous studies.[25] Reactivity in *in situ* carcinomas and in lymph node metastases, however, has not been previously documented. Immunostaining in *in situ* carcinomas is a common finding, and it does not seem to be related to the histologic type (comedo *vs.* noncomedo). Differences between cases with and without invasive components are not significant in our material, but because of the small number of cases without invasion, additional studies are required to complement our data.

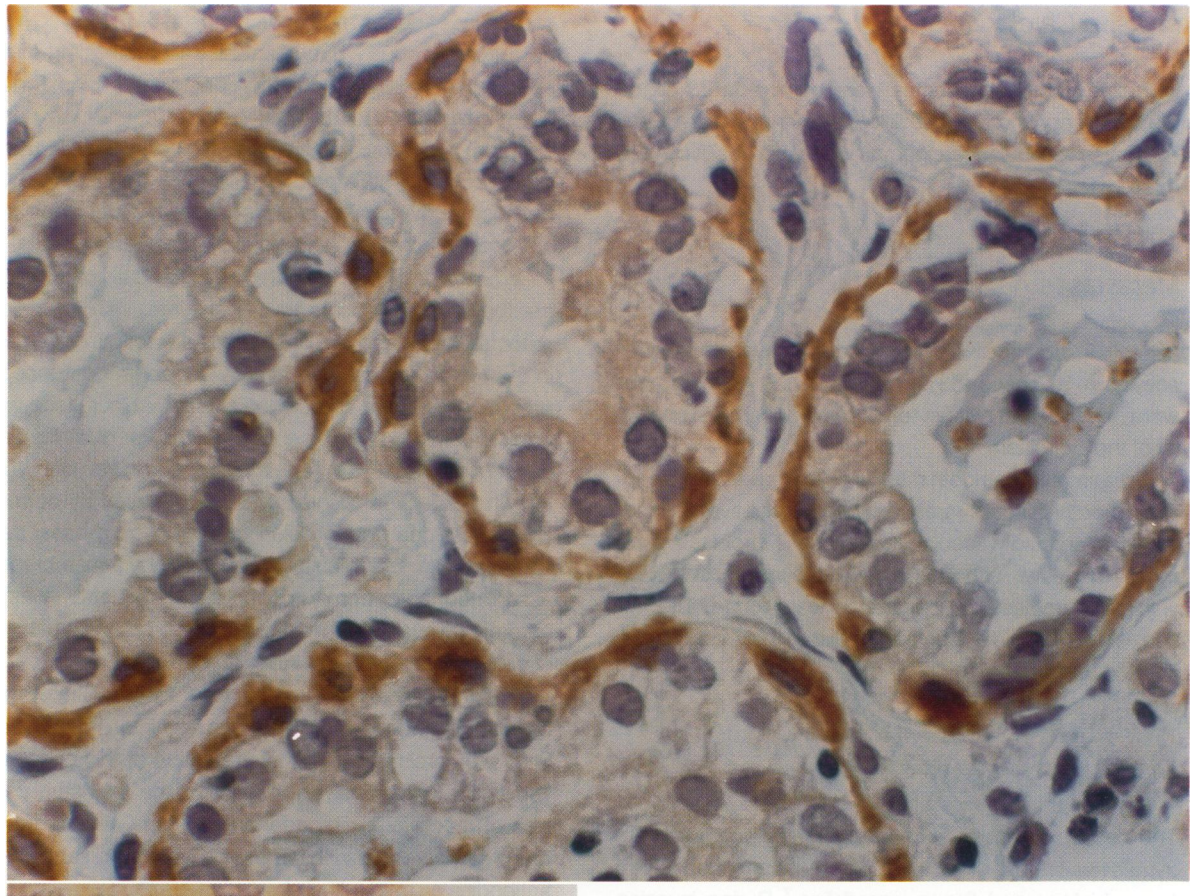
Whether *in situ* or invasive, it seems that tumor cells are the essential source of this enzyme, with no major detectable participation of fibroblasts or other cell types in the tumor stroma. This directly supports the concept proposed by Liotta and coworkers[9,25] that increased expression of type IV collagenase is a marker of malignant conversion. Also, this observation contrasts with the known production of interstitial collagenases by normal fibroblasts, near the invasion front of human basal cell carcinomas,[32,33] or in culture, stimulated by a membrane-associated factor from tumor cells.[28,34] The latter investigators, however, were not able to find production of type IV collagenase by their cultured fibroblasts.[28]

The increased immunoreactivity of epithelial cells in cases of epithelial hyperplasia was less intense than that in *in situ* carcinomas. The interpretation of this observation awaits further analysis. In regard to the inconstant pattern of reactivity in fibroadenomas, some authors have found similar results in these lesions when using anti-actin antibodies as myoepithelial cell markers.[24]

Martinez-Hernandez et al[35] found that an unidentified enzymatic activity was involved in BM collagen turnover in involuting mammary glands. Liotta and coworkers[10,36] have suggested that type IV collagenase could be involved in the breast BM remodeling. Our results of type IV collagenase immunoreactivity in normal breast support the role of the enzyme in these normal processes. Myoepithelial cells seem to be the main cell type involved in this enzymatic activity, although the participation of luminal epithelial cells is increased in terminal ducts.

Although the best-known function of myoepithelium is its contractile ability, a possible role of these cells in BM formation was initially suggested[37,38] and later confirmed *in vitro*.[39,40] Warburton et al[41] found intracellular immunostaining with anti-laminin and type IV collagen antibodies in the basal (myoepithelial-like) cells of the terminal end buds of 7-day-old rats. These authors considered that these cells were actively synthesizing BM components. Now we support the role for myoepithelium in BM collagen turnover, based on the immunoreactivity of this cell type

Figure 2. *Type IV collagenase immunoreactivity in normal and neoplastic breast tissue.* **A (Top left):** *Normal breast. Terminal ducts showing strong immunostaining of myoepithelial cells. (Imm. H_1, 250×)* **B (Top right):** *Intraductal carcinoma. The epithelial cells were markedly positive. (Imm. H_1, 150×)* **C (Bottom left):** *Invasive ductal carcinoma. Notice the intense cytoplasmic immunostaining of the tumor cells. (Imm. MBR, 250×)* **D (Bottom right):** *Lymph node. Metastatic tumor cells are positive, while the lymphocytes are unreactive. (Imm. MBR, 250×).*



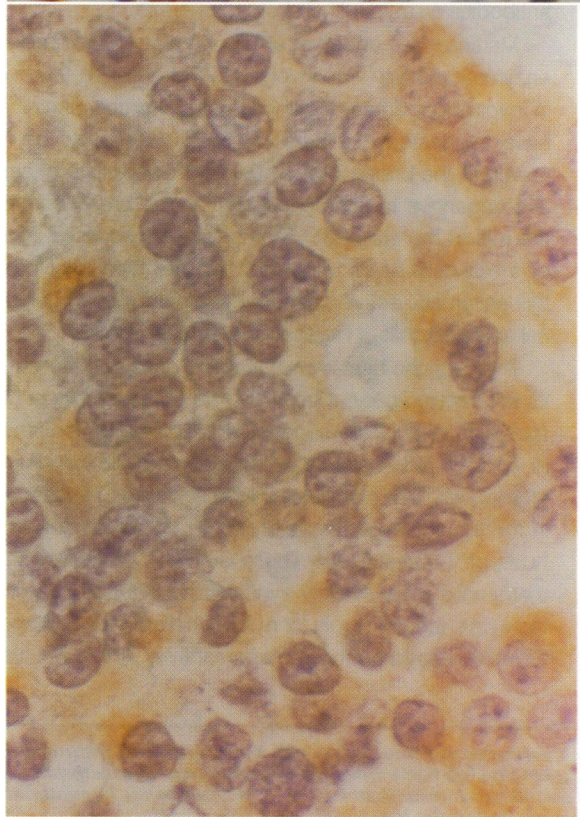


Figure 3. *Lactating breast. The myoepithelial cells show strong positivity. The secretory epithelial cells are negative (Imm. H_1, 250×).*

Figure 4. *Heterogeneous type IV collagenase immunoreactivity in intraductal carcinoma. In this case, the majority of the cells show negative or weakly positive staining (Imm. H_1, 250×).*

for type IV collagenase. The immunostaining of luminal epithelial cells in terminal ducts is not surprising because these cells are also reactive with a myoepithelial cell marker such as KA 1 antibody.[42,43]

In summary, we have used affinity-purified, monospecific antibodies against human type IV collagenase to study the distribution of this enzyme in normal and diseased breast. The results of our study demonstrate that in normal breast tissue type IV collagenase is primarily confined to the myoepithelial cells. We propose that this distribution reflects the participation of these cells in the physiologic turnover of the basement membrane that surrounds breast ducts and ductules. Furthermore, we demonstrate an intense epithelial immunoreactivity with these antibodies in intraductal carcinomas (with and without invasive carcinoma), lobular carcinoma *in situ* (associated with adjacent invasive carcinoma), and invasive carcinomas (ductal and lobular), as well as associated lymph node metastases. Our results clearly show a redistribution in type IV collagenase immunoreactivity from predominantly myoepithelial association in normal and benign breast tissue to an intense epithelial cell staining in malignant breast lesions. This suggests that these antibodies may serve as a useful tool for the evaluation of malignant breast disease.

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